Membranous Glomerulonephritis Induced in the Pig by Antibody to Angiotensin-Converting Enzyme: Considerations on Its Relevance to the Pathogenesis of Human Idiopathic Membranous Glomerulonephritis

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Abstract. In the course of studies on the humoral consequences of swine to primate xenotransplantation, the investigators induced formation of glomerular subepithelial immune deposits and tubular lesions in pigs injected with heterologous antibody to angiotensin-converting enzyme. This study describes the morphology of the lesions, discusses their mechanism, explains their relevance for understanding the pathogenesis of human idiopathic membranous glomerulonephritis, and proposes future directions for investigations.

The etio-pathogenesis of human idiopathic membranous glomerulonephritis (IMGN) remains largely unknown. In laboratory animals, subepithelial immune deposits, the hallmark of the disease, can result from local deposition of circulating immune complexes, from binding of antibodies to nonglomerular antigens planted in the glomerular capillary walls, or from the interaction of antibodies with plasma membrane antigens located in the soles of the epithelial foot processes (1). In the course of studies on the humoral consequences of swine to primate xenotransplantation, we observed by immunohistochemistry that glomerular visceral epithelial cells express angiotensin-converting enzyme (ACE). This finding was unexpected because by immunohistochemical methods ACE is not detectable in the podocytes of rabbits (2), rats, or mice (personal observation). It prompted us to test the nephritogenic potential of ACE antibodies. We observed that pigs injected with heterologous antibody to ACE develop subepithelial immune deposits. We describe this glomerular lesion in the pig, and its relation to other models of glomerulonephritis resulting from the binding of antibodies to antigens of tubular and glomerular epithelial cells (Table 1) (2–15), and discuss their relevance to the pathogenesis of human IMGN.

Materials and Methods

Seven 6-wk-old miniature pigs from Harlan Sprague Dawley (Sinclair Research, Columbia, MO) were used when their body weight was 2.5 to 6 kg. The protocol for animal experiments was approved by the Columbia University Review Board. Goat anti-rabbit lung ACE (GtAACE) (16) and sheep anti-BSA (SABSA) (S. Maruyama, E. Cantu, U. Galili, G. Godman, D. M. Stern, G. Andres. Interaction of baboon anti-α-galactosyl antibody with pig cells and tissues, submitted for publication) sera, and their γ-globulin fractions, were prepared and characterized as described. Pig 1 was infused intravenously with 200 mg/kg GtAACE for 6 h and sacrificed 40 min later to visualize the early antibody binding sites. Pig 2 was injected with 970 mg/kg GtAACE for 4 d and sacrificed 6 h after the last injection, to determine the effect of the longest possible ACE antigen-antibody interaction before additional administration of heterologous proteins would have induced serum sickness. Control pig 3 was injected like pig 2, but with SABSA instead of GtAACE. For the study of long-term consequences, pig 4 was preimmunized with normal goat γ-globulin and infused with 970 mg/kg GtAACE for 4 d and sacrificed 6 h after the last injection, to determine the effect of the longest possible ACE antigen-antibody interaction before additional administration of heterologous proteins would have induced serum sickness. Control pig 3 was injected like pig 2, but with SABSA instead of GtAACE. For the study of long-term consequences, pig 4 was preimmunized with normal goat γ-globulin and infused with 970 mg/kg GtAACE for 4 d, followed by booster injections of normal goat γ-globulin. A renal biopsy was taken after 1 mo, and the pig was sacrificed 3 mo after the beginning of the experiment. Control pig 5 was injected like pig 4, but with SABSA. In pig 6, the right kidney was isolated and perfused ex vivo with 100 mg of GtAACE for 10 min according to a method that we have used previously in rabbits (2). Tissue was obtained at the end of perfusion. Immune aggregates, presumably ACE-anti-ACE complexes, were measured by the Raji cell radioassay (17) in serum samples obtained from pigs 4 and 7, the latter injected with 100 mg of GtAACE over 1 h. Renal tissue was processed for light and electron
microscopy, and for immunohistochemistry (2). Because ACE was denatured by aldehyde fixatives, even at high dilution, and methods for antigen retrieval (18) were not successful, only studies of immunofluorescence could be performed. Serial frozen sections were doubly stained for ACE and laminin, goat IgG and laminin, ACE and goat IgG, and observed by conventional and confocal microscopy. For the study of glomerular visceral epithelial cells, glomeruli were separated by differential sieving, and the epithelial cells were cultured, characterized, and processed as described previously (19). Urinary protein excretion and urinary sediments were examined by conventional methods.

### Results

#### Immunochemistry of Normal Pig Kidney and Cultured Podocytes

In frozen sections, ACE was strongly expressed in the brush border and cytoplasm of proximal tubules, in distal and collecting tubules, in the endothelium of peritubular capillaries and arterioles, and in the epithelium of glomerular capillaries (Figures 1A and 2).

Glomerular visceral epithelial cells were studied after 1 wk of culture, when large polygonal, podocalyxin-positive cells were observed by conventional microscopy and for immunohistochemistry (2). Because ACE was denatured by aldehyde fixatives, even at high dilution, and methods for antigen retrieval (18) were not successful, only studies of immunofluorescence could be performed. Serial frozen sections were doubly stained for ACE and laminin, goat IgG and laminin, ACE and goat IgG, and observed by conventional and confocal microscopy. For the study of glomerular visceral epithelial cells, glomeruli were separated by differential sieving, and the epithelial cells were cultured, characterized, and processed as described previously (19). Urinary protein excretion and urinary sediments were examined by conventional methods.

### Table 1. Subepithelial immune deposits experimentally induced in glomeruli and/or tubules of animals injected with heterologous antibodies to antigens in plasma membranes of glomerular and/or tubular cells, but not in glomerular basement membrane

<table>
<thead>
<tr>
<th>Species</th>
<th>Antigen</th>
<th>Antigen Expression</th>
<th>Immune Deposits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GEC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GEN</td>
</tr>
<tr>
<td>Mouse</td>
<td>CD26</td>
<td>++D</td>
<td>++D</td>
</tr>
<tr>
<td>AA</td>
<td>++D</td>
<td>0</td>
<td>++P</td>
</tr>
<tr>
<td>Rat</td>
<td>Megalin</td>
<td>++CP</td>
<td>++D</td>
</tr>
<tr>
<td></td>
<td>CD26</td>
<td>++D</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit</td>
<td>CD26</td>
<td>++D</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NE</td>
<td>++D</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ACE</td>
<td>0</td>
<td>0/±</td>
</tr>
<tr>
<td>Monkey&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Anti-HBB</td>
<td>0</td>
<td>+ +</td>
</tr>
<tr>
<td>Pig</td>
<td>ACE</td>
<td>++ + D&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+ D</td>
</tr>
</tbody>
</table>

<sup>a</sup> CD26, dipeptidyl peptidase IV; AA, aminopeptidase A; NE, neutral endopeptidase; ACE, angiotensin-converting enzyme; HBB, human renal brush border; GEC, glomerular visceral epithelial cells; GEN, glomerular endothelial cells; D, diffuse; CP, coated pits; P, large, diffuse, and persistent; T, small, focal, and transient; PS, present study.

<sup>b</sup> Including base of foot processes.

<sup>c</sup> Rhesus and cynomolgus.

<sup>d</sup> Localization to the soles of foot processes probable but not proven.

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**Figure 1.** (A) In normal pig kidney, angiotensin-converting enzyme (ACE) is localized in glomerular and tubular epithelial cells, especially in tubular brush border. (B and C) Cultures of normal pig glomeruli with epithelial cells still attached to glomerular remnants (g), or migrated out; in nonpermeabilized cells, ACE is visualized at the surface (B), but after permeabilization, ACE is visualized in the Golgi/endoplasmic reticulum network (C). Magnification: ×600 in A; ×800 in B and C.
were still attached to basement membranes or had migrated out of glomerular remnants. In nonpermeabilized cells, ACE was diffusely distributed at the surface (Figure 1B), but after permeabilization it was visualized in cytoplasm, in a pattern resembling that of proteins in the endoplasmic reticulum/Golgi network (Figure 1C).

**Studies of Pigs Injected with GtAACE or SABSA**

GtAACE was bound to glomerular capillary walls, in a linear pattern (pig 1). By confocal microscopy, goat IgG could be localized to the endothelial side of the glomerular basement membrane. At this time (6 h, 40 min), the kidney was morphologically normal. After 4 d (pig 2), diffuse granular deposits of goat IgG (Figure 3A) and small and focal deposits of pig C3 (Figure 3B) were present along glomerular capillary walls, corresponding to discrete electron-dense deposits in the epithelial filtration slits (Figure 3D). Confocal microscopy showed goat IgG bound to both endothelial and epithelial cells, and colocalized with ACE in the granular deposits (Figure 4, A through C). Goat IgG and pig C3 were also bound to remnants of tubular brush border, and to large phagosomes in proximal tubules. By light microscopy, glomeruli appeared normal; the proximal tubules displayed loss of brush border and flattening of epithelial cells containing many large phagosomes. After 1 mo (pig 4), deposits of pig IgG became evident (Figure 3C), consistent with the development of an autologous phase. After 3 mo, these morphologic and immunohistochemical findings remained unchanged. The kidneys of pigs injected with SABSA (pigs 3 and 5) were normal.

A mild increase of proteinuria (from + to ++) was detected in pigs injected for 4 d with high doses of GtAACE (pigs 2 and 4). The Raji cell radioassay for immune complexes (performed on serum samples obtained before GtAACE infusion) every 5 to 10 min in the first hour after beginning the infusion (pig 7), and every 10 to 20 h during the next 9 d (pig 4), showed no immune aggregates. But after 9 d the values became 10 times higher than normal, concomitant with the development of the autologous phase.

**Immunohistochemistry of Kidneys Perfused ex Vivo with GtAACE or SABSA**

Goat IgG bound in a linear/finely granular pattern was seen by confocal microscopy in glomerular endothelial and epithelial cells of the left kidney of pig 6 perfused with GtAACE, whereas sheep IgG was absent in the glomerular cells of the right kidney, perfused with SABSA.

**Discussion**

The hypothesis that the glomerular subepithelial immune deposits, here described, resulted from local formation and aggregation of immune complexes, is based on the following considerations. (1) ACE is strongly expressed at the surface and within the podocytes and, in much lesser amount, at the surface of glomerular endothelial cells. (2) Shortly after the beginning of intravenous injection of GtAACE, or after *ex vivo* kidney perfusion, goat IgG bound in a linear/finely granular pattern to glomerular endothelial and epithelial cells. Confocal microscopy suggests that ACE is weakly expressed on endothelial cells and, strongly, at the sole of foot processes. (3) On day 4 granular immune deposits were found in the glomerular capillary walls, before formation of detectable immune complexes in the circulation, *i.e.*, too early for their accumulation in glomeruli. (4) Immune aggregates were not revealed in the circulation by the Raji cell radioassay, including those serum samples obtained at frequent intervals during the first hour of injection, when maximum antibody-mediated shedding of immune complexes from extrarenal vascular beds, especially the pulmonary, would be expected to occur (16).

This pig model of membranous glomerulonephritis induced by antibody to ACE bears some similarities to models of membranous glomerulonephritis in other species. The rapid formation and persistence of subepithelial immune deposits are similar to what occurs in mice (3,4) and rabbits (11,12) injected with antibodies to hydrolases which, in the nephron, are mainly localized at the surface of tubular and glomerular epithelial cells. The aggregation of immune complexes, with formation of granular deposits, depends on the mobility of the antigens (20) and extensive antibody cross-linking (13); their persistence results from increased synthesis and secretion of the antigen in response to antibody stimulation, a phenomenon documented in Heymann glomerulonephritis (21). In the pig model described here, and in other models listed in Table 1, both glomerular and tubular lesions are observed.

Because the demonstration that subepithelial immune deposits can be formed *in situ* by antibodies that bind Heymann antigens at the sole of foot processes (20,22,23), the major research effort has been focused on models that, like Heymann nephritis, involve tubular antigens that are also expressed in...
glomerular epithelium. In these models, once the glomerular permeability is increased, the circulating antibodies enter the filtrate and bind and induce injury to the tubules (14,24).

However, in human IMGN, with a few exceptions, anti-brush border antibodies are not demonstrable in patients’ sera, and tubular lesions are absent (1). For this reason, the pig model

Figure 3. (A) Granular deposits of goat IgG, visualized with FITC-rabbit anti-goat IgG, in the glomerular capillary walls, 4 d after injection of Goat anti-rabbit lung ACE (GtAACE) (pig 2). (B) Deposits of pig C3, shown with donkey anti-pig C3 (Sigma), 3 mo after injection of GtAACE (pig 4). (C) Pig IgG visualized in the glomerular capillary walls with FITC-rabbit anti-pig IgG, 1 mo after the injection of GtAACE (pig 4). (D) Electron micrograph showing discrete deposits in the filtration slits (arrows), 4 d after injection of GtAACE (pig 2). Magnification: ×400 in A through C; ×25,000 in D.

Figure 4. Kidney sections double-stained with FITC-rabbit anti-goat IgG (Sigma) and biotinylated goat anti-ACE/Texas Red-avidin (Sigma), and observed by confocal microscopy. The granular deposits of ACE (red, Panel A) and goat IgG (green, Panel B) are colocalized (yellow/orange, Panel C), suggesting that they contain immune complexes (pig 4). Magnification, ×3000.
and others listed in Table 1 may not be directly relevant to human IMGN. This is especially the case for Heymann nephritis, because hydrolases are present on the plasma membranes of human podocytes, but megalin is not (25). Lewis rats, in which megalin is a dominant antigen in podocyte foot processes, develop subepithelial deposits in response to anti-brush border antibodies; however, in rabbits (26,27) (Table 2) and in rhesus and cynomolgus monkeys (15) (Table 1), which lack a dominant tubular antigen in glomeruli, analogous immunizations induce deposits only in the proximal tubules, where circulating antibodies interact with still uncharacterized cytoplasmic antigens “leaking out” or diffusing off epithelial cells. Similar deposits are formed between basement membranes and epithelial cells in the initial segment of the distal nephron (28), and in other organs (29,30) (Table 2), when such “sequestered” antigens, after leaking into the circulation, elicit formation of autoantibodies.

In other models, glomerular subepithelial immune deposits can be produced by autoantibodies to glycoproteins, especially those of the basement membrane, secreted by or leaking out of podocytes, as in rabbits injected with mercuric chloride (31–33) (Table 2) or repeatedly immunized with basement membrane antigens (isolated from the urine) (34) (Table 2). Approximately 3 to 4% of Scripps New Zealand White rabbits spontaneously develop proteinuria and glomerular subepithelial immune deposits containing antibodies that react with epitopes expressed on the soles of rabbit foot processes, and also with epitopes in the peripheral capillary walls of baboon and human glomeruli (35) (Table 2). The antigens of these models—different from those of Heymann nephritis—need to be identified, and the ability of antibodies elicited by them to form glomerular subepithelial deposits should be tested in primates and in isolated perfused human kidneys (15). Moreover, we should examine, using the approach that led to the identification of the megalin complex (6,7,21), the structure, molecular composition, and function of human and nonhuman primate podocytes, especially those of species closer to humans in evolution.

The search for antibodies in serum and in glomeruli of patients with IMGN (36–40) ought to be renewed with more sophisticated methods, using samples from early stages of disease, and should include antibodies made by plasma cells in the spleen and lymph nodes. It is understood that in older glomerular deposits, antibodies with rheumatoid factor or anti-idiotypic activities (37–39) may form secondarily, thereby masking the primary antibody. These may be analogous to anti-Ig antibodies in rats with chronic passive Heymann nephritis (41).

The hypothesis to be tested is that antibodies may react with fixed plasma membrane antigens of the podocytes, or with antigens secreted by, or leaking out of them, as has been presumed to occur in extrarenal organs of some patients with autoimmune diseases (42,43) (Table 2). The rare forms of IMGN associated with antibodies to glomerular (reviewed in references 44 and 45) or tubular (reviewed in reference 46) basement membrane, or with

<table>
<thead>
<tr>
<th>Species</th>
<th>Immunogen, Disease</th>
<th>Site of Immune Deposits</th>
<th>Antibody Specificity</th>
<th>Putative Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>AI, renal tubular BB, or cells</td>
<td>SEP, in renal proximal tubules</td>
<td>Unknown</td>
<td>Ag leaking out</td>
<td>26,27</td>
</tr>
<tr>
<td>Rat</td>
<td>AI, Tamm-Horsfall protein</td>
<td>SEP, in TALHL</td>
<td>Tamm-Horsfall protein</td>
<td>Ag secreted</td>
<td>28</td>
</tr>
<tr>
<td>Mouse</td>
<td>AI, thyroglobulin</td>
<td>SEP, in thyroid follicles</td>
<td>Thyroglobulin</td>
<td>Ag secreted</td>
<td>29</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Vasectomy</td>
<td>SEP, in seminiferous tubules</td>
<td>Acrosome</td>
<td>Ag leaking out</td>
<td>30</td>
</tr>
<tr>
<td>Rabbit</td>
<td>HgCl₂ administration</td>
<td>SEP, in glomeruli and vessel walls</td>
<td>Laminin, type IV collagen, HSPG, etc.</td>
<td>Ag secreted or leaking out</td>
<td>31–33</td>
</tr>
<tr>
<td>Rabbit</td>
<td>AI, urinary basement membrane Ag</td>
<td>SEP, in glomeruli</td>
<td>Unknown basement membrane Ag</td>
<td>Ag secreted or leaking out</td>
<td>34</td>
</tr>
<tr>
<td>Monkey b</td>
<td>AI, HBB</td>
<td>PCT</td>
<td>Unknown</td>
<td>Ag leaking out?</td>
<td>15</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Spontaneous MGN</td>
<td>SEP, in glomeruli</td>
<td>Unknown Ag of GCW</td>
<td>Unknown</td>
<td>35</td>
</tr>
<tr>
<td>Human</td>
<td>Hashimoto</td>
<td>SEP, in thyroid follicles</td>
<td>Thyroglobulin?</td>
<td>Ag secreted or leaking out</td>
<td>42</td>
</tr>
<tr>
<td>Human</td>
<td>Orchitis</td>
<td>SEP, in seminiferous tubules</td>
<td>Unknown (probably sperm)c</td>
<td>Ag leaking out?</td>
<td>43</td>
</tr>
</tbody>
</table>

a Ag, antigen(s); Ab, antibody(ies); AI, active immunization; SEP, subepithelial; TALHL, thick ascending limb of Henle’s loop; HSPG, heparan sulfate proteoglycans; HBB, human renal brush border; PCT, proximal convoluted tubules; MGN, membranous glomerulonephritis; GCW, glomerular capillary wall.

b Rhesus and cynomolgus.

“thin basement membrane disease” (47), may afford useful clues. Sera and renal eluates of two patients with IMGN did not bind to normal human glomeruli (G. Andres, personal observation) (R. T. McCluskey, personal communication) (40). However, in early experiments with passive Heymann nephritis the antibodies did not stain glomeruli, from which it was erroneously assumed that circulating immune complexes had formed (48). These clinical approaches are obviously difficult, because of the scarcity of sera and renal tissues early in the disease; of biopsies of spleen or lymph nodes; and availability of normal kidney for perfusion. Moreover, it is likely that in patients the antigen expression may be upregulated compared with normal kidney, limiting the sensitivity of these techniques. In studies of nonhuman primates (Great Apes excluded), testing of several strains may be necessary to ascertain which alloantigens can be inculpated (49) or whether there is a genetic predisposition as in patients with IMGN (50).

The proposition that all or most human IMGN result from in situ formation of immune complexes with antigens of podocyte origin, such as those depicted here, has not been proven, nor have the pathogenic antigens been identified. Thus, the hypothesis that circulating immune complexes may be the primary pathogenetic agency (51) still remains a valid alternative if we consider that such antibodies may be almost undetectable in the serum in the presence of very large amounts of circulating antigens, such as viruses (52), immunoglobulins (39,40,53,54), or other plasma proteins (55). The search for these “hidden” antibodies could be performed at the cellular level with B cells isolated from spleen or lymph nodes, mindful that in IMGN, antibodies could be performed at the cellular level with B cells or other plasma proteins (55). The search for these “hidden” antigens, such as viruses (52), immunoglobulins (39,40,53,54), serum in the presence of very large amounts of circulating antigens, such as viruses (52), immunoglobulins (39,40,53,54), or other plasma proteins (55). The search for these “hidden” antibodies could be performed at the cellular level with B cells isolated from spleen or lymph nodes, mindful that in IMGN, and in other diseases with immune complexes, antibody production may not be limited to a specific cellular or tissue antigen, but may include antibodies to serum proteins (55). Only directed clinical studies and experimental research in primates can resolve the issues that until now have eluded understanding of the pathogenesis of human IMGN.

Acknowledgments

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